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<b>(54) Title:</b> MONOCLONAL ANTIBODIES TO ELAM-1 AND THEIR USES  <b>(57) Abstract</b>  Compositions and methods are provided for inhibiting inflammatory and other disease responses mediated by ELAM-1. In particular, the present invention provides immunoglobulins which selectively bind functional epitopes on ELAM-1 and block adhesion of leukocytes to activated endothelial cells both <i>in vitro</i> and <i>in vivo</i> .		

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MONOCLONAL ANTIBODIES TO ELAM-1  
AND THEIR USES

10

## BACKGROUND OF THE INVENTION

15           The present invention relates to compositions and methods for treating inflammation and other pathological conditions mediated by intercellular adhesion. In particular, it relates to inhibition of cellular adhesion using novel immunoglobulins that selectively bind functional epitopes on a  
20 cell surface receptor (ELAM-1) involved in intercellular adhesion.

          The vascular endothelium plays a key role in binding certain cells in the blood stream prior to their movement through the vessel wall and into surrounding tissue. For  
25 instance, certain inflammation-triggering compounds such as bacterial endotoxin, tumor necrosis factor, and interleukin 1 act directly or indirectly on the vascular endothelium to promote adhesion of leukocytes and lymphocytes. These cells then move through the blood vessel wall and into areas of  
30 injury or infection. Cellular adhesion to the vascular endothelium is also thought to be involved in tumor metastasis. Circulating cancer cells apparently take advantage of the body's normal inflammatory mechanisms and bind to areas of blood vessel walls where the endothelium is activated.

35           Recent work has revealed that specialized cell surface receptors on endothelial cells, platelets, and leukocytes (designated LEC-CAMs, LECAMs, or selectins) are involved in various intercellular interactions. These

receptors are surface glycoproteins with a lectin-like domain, a region with homology to epidermal growth factor, and a region with homology to complement regulatory proteins (See, Bevilacqua et al., Science, 243:1160 (1989), which is incorporated herein by reference). For instance, a selectin designated LECAM-2 or ELAM-1 has been shown to mediate endothelial leukocyte adhesion to activated endothelial cells, which is the first step in many inflammatory responses. Specifically, ELAM-1 has been shown to bind human neutrophils, monocytes, and the promyelocytic cell line HL-60.

Cell surface receptors of this general class can be expressed in a variety of cells. For example, GMP-140 (also known as PADGEM, LECAM-3 and CD62) is another selectin receptor, which is present on the surface of activated platelets, where it mediates platelet-leukocyte interactions. Similarly, LAM-1 (also known as LECAM-1) is a constitutively expressed cell surface receptor of circulating lymphocytes, and acts as a lymph node "homing" receptor.

Various methods are being developed to block the action of these receptors and thus inhibit cellular adhesion. For instance, monoclonal antibodies directed to ELAM-1 have been proposed as agents to inhibit endothelial-leukocyte adhesion. However, a nexus between antibody inhibition of adhesion and in vivo efficacy remains unclear. Thus, a need exists for evidence of effective treatment for inflammatory diseases using antibodies reactive with ELAM-1.

#### SUMMARY OF THE INVENTION

The present invention provides immunoglobulins capable of binding a functional epitope on ELAM-1 and thereby inhibiting intercellular adhesion in patients. In particular, the immunoglobulins are effective in treating various inflammatory disease responses mediated by ELAM-1, such as septic shock, adult respiratory distress syndrome or wound associated sepsis. A particularly preferred immunoglobulin is secreted by a cell line designated A.T.C.C. Accession No. HB 10591, deposited under the Budapest Treaty on October 30, 1990.

The present invention provides pharmaceutical compositions and methods for treating or diagnosing inflammatory responses in a patient. The compositions are preferably administered intravenously. An effective  
5 therapeutic dosage is between about 1 mg/kg body weight to about 20 mg/kg body weight, preferably between about 5 mg/kg body weight to about 15 mg/kg body weight. The compositions may also comprise targeted liposomes in which the immunoglobulin is embedded. The liposomes may comprise anti-  
10 inflammatory chemotherapeutic agents. The immunoglobulins are typically labelled when used as diagnostic agents.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows prophylactically administered monoclonal  
15 antibodies against ELAM-1 prevent lipopolysaccharide induced death in rats.

Fig. 2 shows therapeutically administered monoclonal antibodies against ELAM-1 prevent lipopolysaccharide induced death in rats.

20 Fig. 3 demonstrates the ability of monoclonal antibodies of the present invention to inhibit lipotechoic acid induced pleuritis in rats.

Fig. 4 shows optimal concentrations of lipopolysaccharide and lipotechoic acid required to induce  
25 neutrophil adhesion to human endothelial cells.

Fig. 5 shows the time course of lipopolysaccharide and lipotechoic acid induced neutrophil adhesion and that this adhesion is inhibited by antibodies to ELAM-1.

Fig. 6 presents a blood clearance profile of a  
30 monoclonal antibody of the present invention, EB3-1.

Fig. 7 demonstrates the effect of EB3-1 and modified forms on lipopolysaccharide induced lethality in rats.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

35 This invention concerns compositions and methods for inhibiting inflammatory and other diseases in which ELAM-1 is involved. Specifically, the invention utilizes immunoglobulins which have the ability to inhibit selectin-mediated adhesion of

the cells in vivo. The immunoglobulins of the present invention selectively bind functional epitopes on ELAM-1 and effectively block adhesion of leukocytes to the vascular endothelium. The present invention also provides methods for  
5 preparing the immunoglobulins and screening assays to identify those that specifically inhibit intercellular adhesion mediated by ELAM-1. In addition, diagnostic and therapeutic uses for these compounds are provided.

As discussed above, selectins are unique  
10 glycoproteins expressed on the surface of a variety of cells. For instance, endothelial leukocyte adhesion molecule 1 (ELAM-1) is inducibly expressed on vascular endothelial cells (Bevilacqua et al., supra and Hession et al., Proc. Nat'l. Acad. Sci., 87:1673-1677 (1990), both of which are incorporated  
15 herein by reference). This receptor has been demonstrated to be induced by inflammatory cytokines such as interleukin I $\beta$  (IL-I $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), as well as bacterial endotoxin (lipopolysaccharide) (see, Bevilacqua et al., Proc. Natl. Acad. Sci., 84:9238-9242 (1987) which is  
20 incorporated herein by reference). These compounds act directly on endothelial cells in vitro to substantially augment polymorphonuclear leukocyte (granulocyte), and monocyte adhesion (Bevilacqua et al., Proc. Natl. Acad. Sci., supra).

Recent work has provided evidence of an  
25 oligosaccharide ligand recognized by ELAM-1. This ligand, known as sialyl Lewis X (SLX), is a terminal structure found on surface glycoproteins and glycolipids of granulocytes. See, Phillips et al., Science, 250:1130-1132 (1990); and Walz et al., Science, 250:1132-1135 (1990).

30 As discussed above, GMP-140 is a membrane glycoprotein of platelet and endothelial secretory granules (Geng et al., Nature, 343:757-760 (1990) which is incorporated herein by reference). Activated platelets which express GMP-140 on their surface are known to bind to monocytes and  
35 neutrophils (Jungi et al., Blood 67:629-636 (1986)), and also to monocyte-like cell lines, e.g., HL60 and U937 (Jungi et al., supra; Silverstein et al., J. Clin. Invest., 79:867-874 (1987)), all of which are incorporated herein by reference.

GMP-140 is an alpha granule membrane protein of molecular weight 140,000 that is expressed on the surface of activated platelets upon platelet stimulation and granule secretion (Hsu-Lin et al., J. Biol. Chem., 259:9121-9126 (1984); Stenberg et al., J. Cell Biol., 101:880-886 (1985); Berman et al., J. Clin. Invest., 78:130-137 (1986)). It is also found in megakaryocytes (Beckstead et al., Blood, 67:285-293 (1986)), and in endothelial cells (McEver et al., Blood, 70:355a (1987)) within the Weibel-Palade bodies (Bonfanti et al., Blood, 73:1109-1112 (1989)). Furie et al. U.S. Patent No. 4,783,330, describe monoclonal antibodies reactive with GPM-140. All of the foregoing references are incorporated herein by reference.

A third selectin receptor is the lymphocyte homing receptor (LHR, also referred to as gp<sup>90</sup> MEL (mouse) or LAM-1 human)). See, Siegelman et al., Science, 243:1165-1172 (1989), Rosen, Cell Biology, 1:913-919 (1989), and Lasky et al. Cell, 56:1045-1055 (1989) all of which are incorporated herein by reference. In addition to lymphocyte homing, LHR is believed to function early in inflammatory responses and to mediate neutrophil binding to the endothelium.

The structure and function of selectin receptors has been elucidated by cloning and expression of full length cDNA encoding each of the above receptors (See, e.g., Bevilacqua et al., Science, supra, (ELAM-1), Geng et al., supra (GMP 140), and Lasky et al., supra (MEL-14)). The extracellular portion of selectins can be divided into three segments based on homologies to previously described proteins. The N-terminal region (about 120 amino acids) is related to the mammalian C-type lectin protein family which includes low affinity IgE receptor CD23. Residues 121-155 are related to proteins containing the epidermal growth factor (EGF) motif. After the EGF domain are three to six tandem repetitive motifs of about 60 amino acids each, related to those found in a family of complement regulatory proteins.

The immunoglobulins of the present invention recognize and selectively bind functional epitopes on ELAM-1 and thereby inhibit intercellular adhesion in in vitro assays. The exemplary immunoglobulins described herein can be utilized

in various standard screening procedures to identify additional immunoglobulins within the scope of the present invention. Moreover, in vivo evidence provided here demonstrates that the claimed antibodies are also effective in treatment of inflammatory conditions mediated by ELAM-1. A "functional epitope" as used herein refers to an antigenic site on an ELAM-1 receptor which is selectively bound by an antibody which antibody substantially inhibits binding of the ELAM-1 ligand to the ELAM-1 receptor and thereby inhibits an inflammatory disease response in a patient. For the purposes of this application, "substantial inhibition" is at least about 60% inhibition, preferably about 70% to about 90%, and more usually about 99% or more (as measured in the in vitro assay as described below).

A number of antibodies have been identified by screening activated endothelial cells. See, e.g., Bevilacqua et al. Proc. Nat'l. Acad. Sci., supra; Poher et al., J. Immunol. 136: 1680 (1986); Graber et al., J. Immunol., 145:819-830 (1990); Leeuwenberg et al., J. Immunol., 156:2110-2114 (1990); Wellicome et al., J. Immunol., 144:2558:2568 (1990); and PCT Publication Nos. WO 90/05786, WO 90/05539 and WO90/13300, all of which are incorporated herein by reference.

The claimed immunoglobulins are suitable for modification using the multitude of techniques available to those skilled in the art for production and manipulation of various immunoglobulin molecules. A few of the possible modifications will be discussed in more detail below.

The basic immunoglobulin structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25kD) and one "heavy" chain (about 50-70kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The C-terminus of each chain defines a constant region primarily responsible for effector functions.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin



genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are  
5 classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

Immunoglobulins may exist in a variety of forms besides whole antibodies, including for example, Fv, Fab, and  
10 F(ab')<sub>2</sub>, as well as in single chains (e.g., Huston et al., Proc. Nat. Acad. Sci. U.S.A., 85:5879-5883 (1988) and Bird et al., Science, 242:423-426 (1988), and Hunkapiller and Hood, Nature, 323:15-16 (1986), all of which are incorporated herein by reference.)

15 Treatment with the protease papain splits the molecule into three fragments, two of which are designated Fab fragments, and the other, the F<sub>c</sub> fragment. The Fab fragments each consist of an antigen binding domain and a C<sub>H</sub>1 domain. Further proteolytic digestion of the Fab fragments releases the  
20 Fv fragment, which consists only of the variable region. The protease pepsin cleaves the heavy chain around the amino acid residues 234 and 333 to yield the F(ab')<sub>2</sub> and pFc' fragments.

The F<sub>c</sub> fragment, which consists of the C<sub>H</sub>2 and C<sub>H</sub>3 domains, is the portion of the immunoglobulin molecule that  
25 mediates various effector functions. Depending upon the heavy chain in the immunoglobulin, a variety of effector functions are present. These include complement fixation, stimulation of B cells, circulatory longevity, and binding F<sub>c</sub> receptors on granulocytes and macrophages prior to phagocytosis See,  
30 generally, Fundamental Immunology, 2d Ed., W.E. Paul, ed., Ravens Press N.Y. (1989), and Winkelhake, Immunochem., 15:695-714 (1978), which are incorporated herein by reference.

Immunoglobulins of the present invention may be produced by a variety of means. The production of non-human  
35 monoclonal antibodies, e.g., murine, lagomorpha, equine, etc., is well known and may be accomplished by, for example, immunizing the animal with an isolated ELAM-1 receptor, activated endothelial cells, or cells transformed with DNA

encoding the ELAM-1 receptor. Antibody-producing cells obtained from the immunized animals are immortalized and screened, or screened first for the production of the desired antibody and then immortalized. For a discussion of general procedures of monoclonal antibody production, see, Harlow and Lane, *Antibodies, A Laboratory Manual* (1988), which is incorporated herein by reference. For methods suitable to raise antibodies to ELAM-1 see, e.g., Bevilacqua et al. *Proc. Nat'l. Acad. Sci.*, supra; Poker et al., *J. Immunol.* 136: 1680 (1986); Graber et al., *J. Immunol.*, 145:819-830 (1990); Leeuwenberg et al., *J. Immunol.*, 156:2110-2114 (1990); Wellicome et al., *J. Immunol.*, 144:2558:2568 (1990); EP Publication No. 408,859 A2; and PCT Publication Nos. WO 90/05786, WO 90/05539 and WO 90/13300, all of which are incorporated herein by reference.

The monoclonal antibodies of the present invention are preferably screened for the ability to inhibit adhesion mediated by ELAM-1 and other selectin receptors in assays such as that described below in Example 1. Ideally, the assays allow large scale in vitro screening of immunoglobulins. Numerous direct and indirect methods for in vitro screening of inhibitors of ligand-receptor interactions are available and known to those skilled in the art. For instance, the ability to inhibit adhesion of ligand-bearing cells, such as PMNs, to cells expressing the particular selectin can be determined. As discussed above, selectin receptor genes have been cloned, thus the genes can be inserted and expressed in a wide variety of cells, such as COS cells, CHO cells and the like. Typically, the test immunoglobulin is incubated with labelled ligand-bearing cells and activated selectin-bearing cells immobilized on a solid surface. Inhibition of cellular adhesion is then determined by detecting label bound to the surface after appropriate washes. In the exemplified assays described below, PMNs and activated human endothelial cells or activated platelets were used.

The immunoglobulins identified by the assays described above are suitable for a variety of pharmaceutical, diagnostic and other applications. In addition to the

treatment and diagnosis of selectin-mediated diseases described more fully below, the immunoglobulins can be used to screen for other monoclonal antibodies that recognize a functional epitope. Such antibodies can be identified by their ability to  
5 block binding of the claimed immunoglobulins to cells expressing a selectin receptor.

The therapeutic efficacy of non-human immunoglobulins in humans, however, is often limited because they have a short serum half-life and induce a human immune response. Thus, it  
10 may be desirable to modify the antibodies to improve therapeutic utility. A variety of strategies are available to improve diagnosis and therapy using monoclonal antibodies, see, Waldmann, Science 252:1657-1662 (1991), which is incorporated herein by reference.

One method suitable for modifying antibodies of the present invention is to transfer the antigen binding regions of the non-human antibodies to portions of human antibodies. For instance, F(ab')<sub>2</sub> fragments can be linked to human constant regions to produce chimeric antibodies. Alternatively,  
20 hypervariable regions can be linked to human framework regions to produce what are referred to here as "humanized" antibodies. Such methods are generally known in the art and are described in, for example, U.S. Patent No. 4,816,397, U.S. Patent No. 4,816,567 and EP publications 173,494 and 239,400, PCT  
25 Publication No. WO/90/07861 and Reichmann, L. et al., Nature, 332:323-327 (1988), all of which are incorporated herein by reference.

Chimeric and humanized antibodies are typically constructed by recombinant DNA techniques from immunoglobulin  
30 gene segments belonging to different species. For example, the variable (V) segments of the genes for a mouse monoclonal antibody may be joined to human constant (C) segments, such as  $\gamma_1$ , and  $\gamma_3$ . A preferred therapeutic chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain  
35 from a mouse antibody and the C or effector domains from a human antibody, although other mammalian species may be used.

Chimeric and humanized antibodies have a number of potential advantages over mouse antibodies for use in human

therapy. For instance, the human immune system may not recognize the C region of the chimeric antibody as foreign, and therefore the antibody response against an injected chimeric antibody should be less than that directed against a totally foreign mouse antibody. In addition, injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of human antibodies of the same class. (Shaw, D. et al., J. Immunol., 138:4534-4538 (1987)). It is possible that injected chimeric antibodies will have a half-life more like that of human antibodies, allowing smaller and less frequent doses to be given.

Another method for producing antibodies of the invention is to isolate DNA sequences which encode a human monoclonal antibody or portions thereof that specifically bind to the human selectin receptor by screening a DNA library from human B cells according to the general protocol outlined by Huse et al., Science, 246:1275-1281 (1989), incorporated herein by reference, and then cloning and amplifying the sequences which encode the antibody (or binding fragment) of the desired specificity.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain variable or hypervariable regions from the claimed monoclonal antibodies. The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate constant regions, such as human gamma heavy chain regions or human kappa light chain regions. One of skill will recognize that, due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below.

The DNA segments will typically further include an expression control DNA sequence operably linked to the chimeric antibody coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions

suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the light chains, heavy chains, light/heavy chain dimers or intact chimeric antibodies may follow.

5           It is well known that native forms of "mature" immunoglobulins will vary somewhat in terms of length by deletions, substitutions, insertions or additions of one or more amino acids in the sequences. Thus, both the variable and constant regions are subject to substantial natural  
10   modification, yet are "substantially identical" and still capable of retaining their respective activities. Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells or long term human B-cells  
15   (Banchereau, et al., Science, 251:70-72 (1991), which is incorporated herein by reference). Suitable source cells for the DNA sequences and host cells for expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection ("Catalogue of Cell Lines and  
20   Hybridomas," Fifth edition (1985) Rockville, Maryland, U.S.A., which is incorporated herein by reference).

          In addition to these naturally-occurring forms of immunoglobulin chains, "substantially identical" modified heavy and light chains can be readily designed and manufactured  
25   utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the chains can vary from the naturally-occurring sequence at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like.  
30   Alternatively, polypeptide fragments comprising only a portion (usually at least about 60-80%, typically 90-95%) of the primary structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., complement fixation activity), while exhibiting lower immunogenicity. In  
35   particular, it is noted that like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities. These may be fused to functional regions from

other genes (e.g., enzymes) to produce fusion proteins (e.g., immunotoxins) having novel properties. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed  
5 mutagenesis (see, Gillman and Smith, Gene, 8:81-97 (1979) and Roberts, S. et al, Nature, 328:731-734 (1987), both of which are incorporated herein by reference).

The nucleic acid sequences of the present invention capable of ultimately expressing the desired chimeric  
10 antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, etc.) and components (e.g., V, J, D, and C regions), as well as by a variety of different techniques. Joining appropriate genomic sequences is presently the most common method of production, but cDNA  
15 sequences may also be utilized (see, European Patent Application Nos. 85102655.8, 85305604.2, 84302368.0 and 85115311.4, as well as PCT Application Nos. GB85/00392 and US86/02269, all of which are incorporated herein by reference).

As stated previously, the DNA sequences will be  
20 expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly,  
25 expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one prokaryotic host useful particularly  
30 for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilis, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors,  
35 which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a

tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, etc, but preferably transformed B-cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen, C. et al., Immunol. Rev., 89:49-68 (1986), which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment may be used for other cellular hosts. See, generally, Sambrook et al., Molecular Cloning: A Laboratory

Manual, 2nd Ed., Cold Spring Harbor Press, (1989), which is incorporated herein by reference.

Once expressed, the whole chimeric antibodies, their dimers, or individual light and heavy chains of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fraction column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982)). Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically or in developing and performing assay procedures, immunofluorescent stainings, and the like (see, generally, Immunological Methods, Vols. I and II, Eds. Lefkovits and Pernis, Academic Press, New York, N.Y. (1979 and 1981)).

The compositions of the present invention comprise monoclonal antibodies which selectively bind to the selectin receptors on cells associated with a number of disorders. For instance, a number of inflammatory disorders are associated with selectins expressed on vascular endothelial cells. The term "inflammation" is used here to refer to reactions of both the specific and non-specific defense systems. A specific defense system reaction is a specific immune system reaction to an antigen. An example of specific defense system reactions includes an antibody response to antigens, such as viruses, and delayed-type hypersensitivity. A non-specific defense system reaction is an inflammatory response mediated by leukocytes generally incapable of immunological memory. Such cells include granulocytes and macrophages. Examples of non-specific reactions include the collection of PMN leukocytes at sites of bacterial infection (e.g., pulmonary infiltrates in bacterial pneumonias and pus formation in abscesses).

Inflammatory conditions treatable with the present invention include, e.g., septic shock, wound associated sepsis, rheumatoid arthritis, post-ischemic leukocyte-mediated tissue damage (reperfusion injury), acute leukocyte-mediated lung injury (e.g., adult respiratory distress syndrome), immune complex mediated tissue (e.g., lung) injury, and chronic inflammatory conditions, including atopic dermatitis and



psoriasis. In addition, tumor metastasis can be prevented by inhibiting the adhesion of circulating cancer cells. Examples include carcinoma of the colon and melanoma.

The antibodies and pharmaceutical compositions thereof of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. Intact immunoglobulins or their binding fragments, such as Fab, F(ab')<sub>2</sub>, etc., are suitable for use in the pharmaceutical composition. In addition, a number of new drug delivery approaches are being developed, the pharmaceutical compositions of the present invention are suitable for administration using these new methods, as well. See, Langer, Science, 249:1527-1533 (1990), which is incorporated herein by reference.

In one embodiment, the antibodies of the present invention can be used to target conventional anti-inflammatory drugs or other agents to specific sites of tissue injury. By using an antibody to target a drug to ELAM-1, such drugs can achieve higher concentrations at sites of injury. Side effects from the conventional anti-inflammatory chemotherapeutic agents can be substantially alleviated by the lower dosages, the localization of the agent at the injury sites and/or the encapsulation of the agent prior to delivery.

The antibodies can be directly or indirectly coupled to the chemotherapeutic agent. The coupling, which may be performed by means, generally known in the art, should not substantially inhibit the ability of the antibody to bind the receptor nor should it substantially reduce the activity of the chemotherapeutic agent. A variety of chemotherapeutics can be coupled for targeting. For example, anti-inflammatory agents which may be coupled include immunomodulators, platelet activating factor (PAF) antagonists, cyclooxygenase inhibitors, lipoxygenase inhibitors, and leukotriene antagonists. Some preferred moieties include cyclosporin A, indomethacin, naproxen, FK-506, mycophenolic acid, etc. Similarly, anti-oxidants, e.g., superoxide dismutase, are useful in treating reperfusion injury. Likewise, anticancer agents, such as

daunomycin, doxorubicin, vinblastine, bleomycin, etc., can be targeted.

The selectin receptor targeting may also be accomplished via amphipaths, or dual character molecules (polar:nonpolar) which exist as aggregates in aqueous solution. Amphipaths include nonpolar lipids, polar lipids, mono- and diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids and salts. These molecules can exist as emulsions and foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions and lamellar layers. These are generically referred to herein as liposomes. In these preparations, the drug to be delivered is incorporated as part of a liposome in conjunction with an antibody of the present invention. When the liposomes are brought into proximity of the affected cells, they deliver the selected therapeutic compositions.

The liposomes of the present invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the bloodstream.

Targeting of liposomes using a variety of targeting agents (e.g., ligands, receptors and monoclonal antibodies) is well known in the art. (see, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044, both of which are incorporated herein by reference). Standard methods for coupling targeting agents to liposomes can be used. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (see, Renneisen, et al., J. Biol. Chem., 265:16337-16342 (1990) and Leonetti et al., Proc. Natl. Acad. Sci. (USA) 87:2448-2451 (1990), both of which are incorporated herein by reference).

Liposome charge is an important determinant in liposome clearance from the blood, with negatively charged liposomes being taken up more rapidly by the reticuloendothelial system (Juliano, Biochem. Biophys. Res. Commun. 63:651 (1975)) and thus having shorter half-lives in

the bloodstream. Liposomes with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. Liposomes which can be maintained from 8, 12, or up to 24 hours in the bloodstream provide sustained release of the anti-inflammatory agents. Serum half-life may also be important when the liposome or antibody is labelled to provide for in vivo diagnostic imaging.

A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028, incorporated herein by reference. One method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous solution of the targeted drug and the targeting component (antibody) and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

The hydration medium contains the targeted drug at a concentration which is desired in the interior volume of the liposomes in the final liposome suspension. Typically the drug solution contains between 10-100 mg/ml in a buffered saline. The concentration of the antibody is generally between about 0.1 - 20 mg/ml.

Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The

liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

Even under the most efficient encapsulation methods, the initial sized liposome suspension may contain up to 50% or more drug and targeting agent in free (non-encapsulated) form. Therefore, to maximize the advantages of liposomal targeted drug, it is important to remove free drug and targeting agent from the final injectable suspension. Several methods are available for removing non-entrapped compound from a liposome suspension. In one method, the liposomes in the suspension are pelleted by high-speed centrifugation leaving free compound and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, then resuspending the concentrated liposomes in a drug-free replacement medium. Alternatively, gel filtration can be used to separate large liposome particles from solute molecules.

Following treatment to remove free drug and/or targeting agent, the liposome suspension is brought to a desired concentration for use in intravenous administration. This may involve resuspending the liposomes in a suitable volume of injection medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration. The liposome-ligand preparation may be administered as described below.

As discussed above, the pharmaceutical compositions (comprising targeted liposomes or free antibodies) are particularly suitable for parenteral administration. The composition will commonly comprise a solution of the antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate

physiological conditions such as pH-adjusting and buffering agents, tonicity (e.g., isotonic) adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intramuscular injection could be made up to contain 1 ml sterile buffered water, and 50 mg of antibody. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Sciences, 17th Ed., Mack Publishing Company, Easton, Pennsylvania (1985), which is incorporated herein by reference.

The antibodies of this invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss (e.g., with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions containing the present antibodies or a cocktail thereof can be administered for the prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient in an amount sufficient to cure or at least partially arrest the infection and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the

disease and the general state of the patient's own immune system, but generally range from about 1 mg/kg body weight to about 20 mg/kg body weight, preferably between about 5 mg/kg body weight to about 15 mg/kg body weight. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases, in view of the minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by human chimeric antibody forms of this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally in the ranges described above.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the patient.

The antibodies of the present invention may also be used for diagnostic purposes, such as identifying areas of inflammation. For diagnostic purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the antibody, such as antibodies specific for the particular immunoglobulin constant region. Alternatively, the antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluorescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens),

etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

In diagnostic applications, compositions containing the immunoglobulins or a cocktail thereof, are administered to a patient suspected of having an inflammatory disease state. Alternatively, the efficacy of a particular treatment can be monitored. An amount sufficient to accomplish this is defined to be a "diagnostically effective dose." In this use, the precise amounts will depend upon the patient's state of health and the like.

Kits can also be supplied for use with the subject antibodies. Thus, the subject antibody composition of the present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The antibodies, which may be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of active antibody, and usually present in total amount of at least about 0.001% wt. based again on the antibody concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% wt. of the total composition. Where a second antibody capable of binding to the chimeric antibody is employed in an assay, this will usually be present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the antibody formulations described above.

The following examples are offered by way of illustration, not by limitation.

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#### EXAMPLE 1

This example shows the ability of monoclonal antibodies of the present invention to block adhesion of neutrophils (PMNs) to activated vascular endothelial cells in

an intercellular adhesion assay. The assay procedure was as follows:

Human umbilical cords were obtained following delivery. Sections of cord 20-40 cm in length and free of clamp marks were used. Under sterile conditions, one end of the cord vein was cannulated and connected to a 3-way stopcock and syringe. The cord was flushed with Ca<sup>+</sup>/Mg<sup>+</sup> free phosphate buffered saline (PBS) to remove blood and clots. The opposite end of the cord was then cannulated and connected to a 2-way stopcock, followed by a brief rinse with collagenase (Boehringer Mannheim) 1 mg/ml and the 2-way stopcock was closed. The cord was then filled with collagenase, air was removed and the 3-way stopcock was closed. The cord was placed in a sterile chamber and incubated at 37°C for 15-20 minutes. After incubation, the cord was flushed with 30 ml Ca<sup>++</sup> and Mg<sup>++</sup> containing PBS, and the eluted endothelial cells were collected in a sterile tube containing 5 ml EGM-UV medium (Clonetics) and 10% FCS to inhibit further collagenase activity. The cells were centrifuged and resuspended in complete medium containing EGM-UV, 10 ng/ml epidermal growth factor, 1 ug/ml hydrocortisone, Gentamicin, Amphotericin-B and 10% FCS. A tissue culture flask of appropriate size (according to length of cord segment) was coated with .1% gelatin (endotoxin free, isolated from bovine epidermis) for 15 min. at RT and the excess was removed. The endothelial cell suspension was plated in the flask and placed in a 5% CO<sub>2</sub> incubator. The medium was changed after 16 hrs. when the cells appeared to be adherent. The cells were passaged when sub-confluent (75%). To passage the cells, the medium was removed by vacuum suction and the flask is rinsed 2 times with HEPES (10 mM) buffered saline to remove FCS, 1-2 ml of 0.025% trypsin EDTA was then added to the flask and it was observed under an inverted microscope until the endothelial cells were in suspension (30 sec. - 1 min.). Fresh complete medium was then added to the flask and it was split 1:2 or 1:3 into new gelatin coated flasks.

To establish endothelial cell monolayers on 96 well assay plates, the plates were coated with gelatin as described above. Endothelial cells from cultures that were passaged 3



times were harvested from flasks using 0.025% trypsin as above and plated at  $5 \times 10^4$ /well. The cells were allowed to grow to confluence. Medium was changed one time following plating.

To induce ELAM-1 expression, the medium was removed  
5 from the wells a few wells at a time and replaced with 0.05 ml of fresh complete medium (in control wells) or of fresh complete medium containing 30 ug/ml rIL-1 $\beta$  (in test wells). The plates were returned to the incubator for 4 hrs. These are referred to as stimulated endothelium.

10 Neutrophils (PMNs) were prepared from whole blood. 50 ml of whole blood was drawn from a volunteer donor into heparinized vacutainer tubes. Each 25 ml of blood was layered over 15 ml of "Mono-Poly Resolving Medium" (Flow Labs). The tubes were centrifuged at 20°C for 25 min. at 2000 rpm in an  
15 RT6000 centrifuge and then the speed was increased to 2500 rpm for a further 25 min. The PMN layer (the lower of two floating cell layers) was removed and placed in a clean 50 cc centrifuge tube. 30 ml of Hanks Balanced Salt Solution (HBSS) (Gibco) containing 20 mM HEPES (Gibco) and 0.2% Glucose (Fisher) was  
20 then added to each tube. The tubes were centrifuged at 3000 rpm for 3 min. at room temperature and resuspended in the HBSS/HEPES/glucose buffer 3 times and counted using a hemacytometer.

To perform the assay, the assay plate was removed  
25 from the incubator and the adherent cells were washed X2 with HBSS/HEPES/glucose + 5 mg/ml Bovine Serum Albumin. 100 ul of each monoclonal antibody to be tested was added to assay plate wells. The antibody was incubated on the plates for 20 min.  $5 \times 10^5$  PMN were added to the wells in 50 ul. The plates were  
30 then incubated for 6 min. at room temperature. Non-adherent cells were removed from the wells by inverting the plate over the sink and addition of 200 ul of medium using a multichannel pipette 4 times. The last wash was removed from the wells and 50 ul of Solubilization buffer was added. This consisted of  
35 citrate buffer (24.3 ml of 0.1 M Citric acid, 10.5g/500 ml + 25.7 ml of 0.2 M dibasic sodium phosphate, 14.2g/500 ml and SQ H<sub>2</sub>O to 100 ml) containing 0.1% NP-40 detergent. The plate was incubated with gentle mixing for 10 min. and then 50 ul of OPDA

(8 mg o-phenylene-diamine, Sigma cat. # P-1526, 8 ul of 30%  $H_2O_2$  and 10 ml of SQ  $H_2O$ ) was added to each well. The plates were incubated 15 min. at RT and then 15 ul of 4N  $H_2SO_4$  was added to each well to stop the reaction. A reagent blank was prepared by mixing 50 ul of solubilization buffer, 50 ul of OPDA solution and 15 ul of 4N  $H_2SO_4$ . 100 ul of supernatant was removed from each well and transferred to a flexible ELISA assay plate (Falcon). The plate was scanned at 492 nm within 30 min.

In addition, carcinoma cells, colo 205 (ATCC No. CCL 222), which are known to express SLX were used. Since these cells do not bear  $F_c$  receptors, cross-linking by the monoclonal antibodies should not occur. The assay procedure was the same as that described above for PMNs. The cells, however, were labelled with  $^{51}Cr$  (50  $\mu Ci$ /cell for 30 min.). Adhesion was detected using a gamma counter following standard procedures.

The results presented in Table 1 below show that monoclonals of the present invention effectively block adhesion of PMNs and Colo 205 cells to activated endothelial cells. The prior art monoclonal antibody H18/7 (described in PCT Publication No. WO 90/05539, *supra*) was included as a control. The amount of binding in the absence of antibodies was arbitrarily assigned a value of 1.0. Antibodies that promoted binding in the assays have a value greater than 1, whereas antibodies that inhibited binding have a value less than 1.

Table 1  
Binding of cells to HUVECs  
in the presence or absence of antibodies to ELAM-1

	<u>Mab</u>	<u>Subclass</u>	<u>PMNs</u>	<u>Carcinoma (Colo 205)</u>
30	None		1.00	1.00
	EB1-5	IgG <sub>1</sub>	1.42	0.08
	ENB1-6	IgG <sub>1</sub>	1.64	0.76
35	EB1-7	IgG <sub>1</sub>	1.48	0.10
	EB3-1	IgG <sub>3</sub>	0.18	0.12
	EB3-2	IgG <sub>3</sub>	0.3	0.12
	EBM-2	IgM <sub>3</sub>	0.21	0.12
40	EBM-4	IgM	0.28	0.10
	H18/7	IgG <sub>2</sub> a or b	1.20	0.16

## EXAMPLE 2

This example presents data from competitive inhibition assays used to compare the epitope recognized by monoclonal antibodies of the present invention with the epitope recognized by a monoclonal antibody previously identified as binding ELAM-1 (H18/7). To do this, H18/7 was biotinylated and HRP-avidin staining was detected by solid-phase ELISA on IL-1 activated endothelial cells, according to standard procedures (see, e.g., Harlow and Lane, supra). The values given are absorbance at 492 nm.

As the results in Table 2 indicate, a range of inhibition of H18/7 binding was found.

Table 2

<u>Blocking Mab</u>	<u>Biotinylated Mab</u>	<u>HRP-Avidin</u>
Control	H18/7	1.175
H18/7	H18/7	0.309
EB1-1	H18/7	1.021
EB3-1	H18/7	0.830
ENB1-6	H18/7	0.346

## EXAMPLE 3

This example shows the ability of monoclonal antibodies of the present invention to block adhesion of neutrophils (PMNs) to activated platelets (bearing GMP-140) in intercellular assays. The results of these assays are presented in Table 3, which shows the relative adhesion of PMNs or Colo 205 cells to activated platelets in the presence of these monoclonal antibodies of the present invention. The assay procedure was as follows:

Platelets were obtained from a normal human donor and washed free of plasma proteins in the presence of  $\text{PGE}_1$  (100nM) (see Polley and Nachman, J. Exp. Med., 158:603 (1983), which is incorporated herein by reference). They were then activated with thrombin (0.25U/ml;  $2 \times 10^8$  platelets per ml) for 20 min at room temperature without stirring.

Two assays were used for assessing GMP-140 - mediated adhesion to activated platelets: a plate assay and a fluid phase assay. The plate assay was a modification of the endothelial cell-neutrophil adherence assay previously described (Dobrina, et al., Immunology, 67:502 (1989), which is

incorporated herein by reference). A suspension of platelets (300  $\mu$ l;  $10^8$ /ml) was applied to each well of a 48 well plate which had previously been coated with 0.1% gelatin. The plate was incubated at 37°C for 15 min, then centrifuged for 2 min at 90xg and washed twice with phosphate-buffered saline (PBS) to remove non-adherent platelets. To block platelet  $F_c$  receptors 300  $\mu$ l of heat-aggregated IgG (20 $\mu$ g/ml) was added to each well and the plate was allowed to stand at room temperature for 20 min. The plate was washed once with PBS and then 300  $\mu$ l of the monoclonal antibody to be tested was added to each well of the plate and the plate was allowed to stand at room temperature for a further 20 min. Meanwhile, neutrophils were isolated from whole human blood by the method described above. They were radiolabelled with  $^{51}\text{Cr}$  (450  $\mu$ Ci added to  $3 \times 10^6$  cells in 300  $\mu$ l and incubated for 60 minutes at 37°C), washed three times in RPMI containing 10% fetal calf serum and resuspended to  $2 \times 10^6$ /ml. 50  $\mu$ l of this suspension was added to each well of the assay plate. The plate was centrifuged for 2 min at 90xg and then allowed to stand for 5 min at room temperature. Unbound cells were removed by 3 washes with PBS and the remaining bound cells were removed from the plate with an SDS-containing buffer and then processed for radioactive counting.

The fluid phase assay was performed by a modification of that described (see Larsen et al., Cell, 63:467 (1990) which is incorporated herein by reference. 20  $\mu$ l of the activated platelets ( $2 \times 10^8$ /ml) were placed in an Eppendorf tube. 20  $\mu$ l of heat-aggregated IgG was added and after mixing, the tubes were allowed to stand at room temperature for 20 min. 20  $\mu$ l of the monoclonal antibody was added and the tubes were allowed to stand for a further 20 min at room temperature. Meanwhile, PMNs were prepared as above (without radiolabelling) and were diluted to  $2 \times 10^6$ /ml. 20  $\mu$ l of this suspension was added to each tube and after mixing the tubes were allowed to stand at room temperature for 20 min. Adhesion was then evaluated microscopically and was scored as the per cent of the test cells which had bound two or more platelets.

In addition, assays were performed using cells derived from colon carcinoma, Colo 205 (ATCC No. CCL 222),

which are known to express a ligand recognized by selectin receptors. The assay procedure used was the same as that used with PMNs.

Table 3

	<u>MAB</u>	<u>PMNs</u>	<u>Carcinoma (COLO 205)</u>	<u>Precipitates Purified GMP-140</u>
5	None	+	+	...
	GBI-1	-	-	+
	GBI-2	-	-	+
10	GNBI-3	+	+	+

EXAMPLE 4

This example demonstrates the efficacy of mAb EB3-1 in an animal model of lipopolysaccharide-induced death. A rat system was chosen because EB3-1 has been shown to cross-react with the rat equivalent to ELAM-1.

LPS from E. coli 0111:B4 (Sigma, Lot #36F4019) was prepared fresh from a single lot one day prior to use by dissolving in sterile, pyrogen-free saline at a concentration of 5 mg/ml. The solution was sonicated on ice for 30 seconds using a Tekmark Sonic disrupter. Just prior to use, the material was sonicated a second time for 30 seconds.

Female Lewis rats' weighing 200 g (+- 10g) were purchased from Charles River Breeding Labs and held for at least 7 days after receipt (for adaptation). Groups of 10 animals were used, unless otherwise noted. All reagents were injected parenterally via the tail vein at 0.5-1.0 ml/kg. As negative controls, animals received either sterile, LPS-free saline, or a murine IgG3k myeloma protein (J606, low pyrogen - < 2 ng/mg protein).

The EB3-1 dose/schedule protocols were arrived at empirically from the pharmacokinetic data we obtained with EB3-1 prophylactically administered to rats. A "minimal" LD<sub>100</sub> dose of LPS was determined to be 7.5 mg/kg for these rats.

In one experiment, rats were treated with 10 mg/kg P6E2 one hour before the LPS challenge. A boost dose (1.0 mg/kg) was administered 3 hours after the challenge. 4/10 treated animals survived the LPS challenge. In contrast, all 10 (saline-injected) controls died. At the twenty-four hour

observation period, the survivors showed few of the clinical signs characteristic of LPS-treated animals.

We next tried doses of EB3-1 which were (1) 2-fold higher and (2) an order of magnitude lower than the 10 mg/kg dose used in the first experiment. The results show that EB3-1 had a significant effect: 80% of the animals survived at the 10 mg/kg dose (Figure 1). Note that one animal survived in the saline control group - suggesting that this group of animals was not "hit" as hard by the LPS challenge as were the animals in the previous experiment.

Another study was performed to demonstrate the therapeutic value of EB3-1. Animals received the 10 mg/kg iv bolus dose of EB3-1 at 1 hour before or 2, 4 or 6 hours after the LPS challenge.

Once again, 1 of 10 animals survived in the saline-treated group, as well as in a group treated with 10 mg/kg J606 myeloma protein at T=-60 minutes (Figure 2). However, EB3-1 had a significant protective effect even when administered 2 or 4 hours after LPS.

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### Conclusion

The protection seen with Cytel mAb EB3-1 demonstrates that the antibodies of the present invention are both prophylactically and therapeutically useful in treating inflammatory disease responses mediated by ELAM-1.

### EXAMPLE 5

This example demonstrates the effectiveness of purified EB3-1 antibody to inhibit an inflammatory response induced by lipotechoic acid (LTA) in the rat pleural cavity. The ability of LTA to induce pleuritis has been established with a linear relation of dose concentration to neutrophil infiltration. LTA has also been shown to activate endothelium via ELAM-1 to adhere neutrophils in vitro (see, Example 6, below).

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In this experiment we induced pleuritis in the rat by bilateral injections of LTA into the pleural cavity. In test animals this was followed by a tail vein injection of EB3-1

mAb. The cavities were flushed after 4 hours and evaluation made on the neutrophil concentration of the exudates.

Procedure:

- 5 1. LTA (Sigma Cat #L-2515 Lot 89F4061) was prepared at 2 mg/ml in sterile endotoxin and pyrogen free saline and stored at -70°C. This was made into a 750 µg/ml stock solution by dissolving 1.41 ml LTA 2.34 ml saline. Final concentration 300 µg/400µl/rat.
- 10 2. EB3-1 purified ascites (Lot 6) was 4.5 mg/ml was diluted in DPBS to a final concentration of 3.33 mg/ml. The dose delivered per rat was 600 µl containing 2 mg (10 mg/Mg) sonicated 1.5 min.
- 15 3. Nine three month old female Lewis rats, weighing about 195 grams were used. All were sedated by metaphane inhalation.
4. The rats were divided Into three groups.
  - 20 A. Control n=1 : Received 200 µl of DPBS on each side of the pleural cavity. Received 600 µl sterile, pyrogen and endotoxin free saline via tail vein injection one hour after pleural injection.
  - B. LTA and EB3-1 treated : n=4 Received 150 µg LTA in 200 µl DPBS on each side of the pleural cavity. Received 600 µl EB3-1 (10 mg/Kg, 2 mg total) via tail vein injection one hour after pleural injection.
  - 25 C. LTA only treated : n=4 Received 150 µg LTA in 200 µl DPBS on each side of the pleural cavity. Received 600 µl DPBS via tail vein injection one hour after pleural injection.
- 30 5. The rats were injected between the third and fifth ribs using a 30 gauge needle, blunt tip, on a 1 cc syringe. The tail vein injections were done by first heating the rats under a heat lamp to make veins more prominent, and delivered using a 26 gauge needle on a 3 cc syringe.
- 35 6. The rats were re-caged for 3.5 hours after tail vein injection, 4.5 hours total time.
7. The rats were sacrificed by metaphane inhalation.

8. The peritoneal cavity was opened to expose the diaphragm. A small incision was made on each side of the diaphragm and each side was rinsed with 1 ml heparinized DPBS using a blunt tip 1 cc syringe (no needle) and combined.
- 5 9. The volume of each was noted, the cells counted and pelleted. They were suspended in 100  $\mu$ l saline and a slide smear made of each to be stained with different quick stain for a differential analysis.

10 The results of this experiment are presented in Figure 3, which shows that EB3-1 is effective in inhibiting LTA induced pleuritis. The antibody inhibited PMN migration into the pleural cavity by >80%.

#### 15 EXAMPLE 6

This example demonstrates that the bacterial products Lipopolysaccharide (LPS) and Lipotechoic acid (LTA) induce expression of ELAM-1 on vascular endothelial cells. To do this, the ability of human neutrophils (PMNs) to bind to  
20 cultured human endothelial cells was measured following treatment in vitro with various concentrations of LPS and LTA.

#### Procedure

1. Stock solutions of LPS (Lot # 36F4019) and LTA (Sigma,  
25 # L-2515, Lot 89F4061) were prepared by sonication at 1 mg/ml in saline immediately before use. The stock solutions were diluted in fresh EGM-UV medium (Clonetics) to make working concentrations at: 100 ug/ml, 50 ug/ml, 25 ug/ml, 12 ug/ml, 6 ug/ml, 3 ug/ml, 1.5 ug/ml, 0.75 ug/ml,  
30 0.37 ug/ml, 0.19 ug/ml, and .09 ug/ml. One 96 well Costar cluster dish containing HUVEC cord #115 passage 4 grown to confluence on gelatin, was removed from the incubator. The medium in each well was removed with a pasteur pipette and replaced either with 0.2 ml fresh EGM-UV medium  
35 (Clonetics) or with the same volume of working dilutions of LPS or LTA. Each dilution was assayed in triplicate.
2. The plate was returned to a 37°C incubator with 5% CO<sub>2</sub> for 4 hrs.



3. Neutrophils (PMNs) were prepared as in Example 1. PMNs were maintained at room temperature throughout the preparation.  $1.04 \times 10^8$  PMN were recovered and were resuspended to  $6 \times 10^5/25$  ul in the same buffer but containing 5 mg/ml bovine serum albumin.
4. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed two times with RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA). After the second wash the wells were refilled with 100 ul of the same buffer.
5. 0.025 ml of cell suspension was added to each well on the stimulated assay plate.
6. The plates were incubated for 5 min at 37°C.
7. Unbound cells were removed from the wells of the assay plates by systematic resuspension using a p200 multichannel pipette followed by addition and removal of 0.2 ml of medium.
8. All of the medium was removed from the wells and 50 ul of solubilization buffer was added. This consisted of citrate buffer (24.3 ml of 0.1 M Citric acid, 10.5 g/500 ml + 25.7 ml of 0.2 M dibasic sodium phosphate, 14.2 g/500 ml and SQ H<sub>2</sub>O to 100 ml) containing 0.1% NP-40 detergent.
9. The plate was incubated on a rotary shaker for 10 min and then 0.05 ml of OPDA solution [8 mg o-phenylene-diamine, Sigma cat# P-1526, 8 ul of 30% H<sub>2</sub>O<sub>2</sub>, and 10 ml of citrate buffer (as above)] was added to each well. The reaction was allowed to develop for 15 min and then 25 ul of 4N H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction.
10. A reagent blank was prepared by mixing 100 ul volumes of the solubilization buffer and the OPDA solution with 50 ul of 4N H<sub>2</sub>SO<sub>4</sub>.
11. 100 ul of supernatant was removed from each of 2 wells and transferred to a flexible ELISA assay plate (Falcon). The plate was scanned spectrophotometrically at 492 nm within 30 min.

The results of this experiment are presented in Fig. 4, which shows that both LPS and LTA induce neutrophil adhesion

molecules to human endothelial cells. LPS was most effective between 0.2 and 5 ug/ml. LTA was most effective between 5 and 50 ug/ml. At optimal concentration of each compound, LPS induced 2-3 fold greater neutrophil adhesion than LTA.

5

#### Example 7

This example demonstrates that neutrophil adhesion to LPS and LTA activated endothelium seen in Example 6 is mediated by ELAM-1. The optimal time of activation of endothelium with each endotoxin was also investigated. We measured the ability of human neutrophils (PMNs) to bind to LTA and LPS activated, cultured human endothelial cells at various times following addition of the activating agents, in the presence or absence of EB3-1.

15

#### Procedure

1. Stock solutions of LPS (lot #36F4019) and LTA (Sigma, # L-2515, Lot 89F4061) were prepared by sonication at 1 mg/ml in saline immediately before use. The stock solutions were diluted in fresh EGM-UV medium (Clonetics) to make working concentrations at: 10 ug/ml of LTA and 0.2 ug/ml LPS. One 48 well Costar cluster dish containing HUVEC cord #117 passage 4 grown to confluence on gelatin, was removed from the incubator. At each of 8 time points, T=0, 2 hrs, 3 hrs, 4 hrs, 5 hrs, 6 hrs, 8 hrs, 18 hrs, and 24 hrs, the medium in each of 6 wells was removed with a pasteur pipette and replaced with 0.5 ml fresh EGM-UV medium (Clonetics) containing the working dilutions of LPS (3 wells) or LTA (3 wells).
2. The plate was returned to a 37°C incubator with 5% CO<sub>2</sub> for 4 hrs.
3. Neutrophils (PMNs) prepared as in Example 1. PMNs were maintained at room temperature throughout the preparation. They were resuspended to 2 x 10<sup>6</sup>/50 ul in the same buffer but containing 5 mg/ml bovine serum albumin.
4. The stimulated HUVEC assay plate was removed from the incubator and the wells were washed two times with RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA). After

- the second wash the wells were refilled with 300 ul of the same buffer. The medium was again removed from one well of each triplicate and replaced with 300 ul of hybridoma culture supernatant containing EB3-1 mAb. The plate was
- 5 incubated with the antibody for 10 min. at 37°C and then removed from the incubator.
5. 0.05 ml of PMN suspension was added to each well on the stimulated assay plate.
6. The plates were incubated for 5 min at 37°C.
- 10 7. Unbound cells were removed from the wells of the assay plates by systematic resuspension using a repeat delivery pipette followed by addition and removal of 0.3 ml of medium.
8. All of the medium was removed from the wells and 200 ul of
- 15 solubilization buffer was added. This consisted of citrate buffer (24.3 ml of 0.1 M Citric acid, 10.5 g/500 ml + 25.7 ml of 0.2 M dibasic sodium phosphate, 14.2 g/500 ml and SQ H<sub>2</sub>O to 100 ml) containing 0.1% NP-40 detergent.
9. The plate was incubated on a rotary shaker for 10 min and
- 20 then 0.2 ml of OPDA solution [8 mg o-phenylene-diamine, Sigma cat# P-1526, 8 ul of 30% H<sub>2</sub>O<sub>2</sub> and 10 ml of citrate buffer (as above)] was added to each well. The reaction was allowed to develop for 15 min and then 50 ul of 4N H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction.
- 25 10. A reagent blank was prepared by mixing 100 ul volumes of the solubilization buffer and the OPDA solution with 50 ul of 4N H<sub>2</sub>SO<sub>4</sub>.
11. 100 ul of supernatant was removed from each of 2 wells and transferred to a flexible ELISA assay plate (Falcon). The
- 30 plate was scanned spectrophotometrically at 492 nm within 30 min.

The results of this experiment are shown in Fig. 5, which shows LPS and LTA induction of neutrophil adhesion

35 molecules on human endothelial cells peaked between 4 and 6 hrs of incubation with the endothelial cells. This time course is consistent with that previously shown for the induction of ELAM-1 by TNF and IL-1 $\beta$ . At each time point tested, the PMN

adhesion could be completely inhibited by anti-ELAM-1 mAb, showing that LPS and LTA both induce ELAM-1 expression on human endothelium. The expression of ELAM-1 induced by these compounds rapidly decreased after 6 hrs of exposure  
 5 demonstrating kinetics similar to those of IL-1 $\beta$  activation.

#### Example 8

This example demonstrates that the ability of EB3-1 to inhibit LPS induced lethality is not dependent upon  
 10 complement fixation. First, experiments to determine the relative blocking efficiency of EB3-1 F(ab')<sub>2</sub> fragments and Acid treated EB3-1 were carried. It has been previously shown that treating intact IgG<sub>3</sub> at pH 2.5 for 60 minutes permanently destroys complement fixing abilities. (Winkelhake, J. et al.,  
 15 1980, J. Biol. Chem. 2822-2828, incorporated herein by reference.)

The adhesion of <sup>51</sup>Cr labeled HL-60 cells to IL-1 $\beta$  activated endothelial cells was measured in the presence of titrated amounts of two preparations of EB3-1 mAb (acid treated  
 20 Ig and F(ab')<sub>2</sub>). 50% inhibition of binding was achieved with F(ab')<sub>2</sub> at about 0.5 ug/ml. About 2ug/ml of the acid treated Ig was required to give equivalent inhibition of adhesion.

We next radioiodinated the two modified forms of EB3-1 and determined half-lives to be:

	$\alpha$ -phase (min)	$\beta$ -phase (min)
unmodified EB3-1	2	356
30 pH2.5 treated EB3-1	3.2	408
EB3-1 F(ab') <sub>2</sub>	1.3	136

A blood clearance profile of EB3-1 is presented in  
 35 Figure 6. Based on these data and area-under-curve calculations, we used the two modified forms of EB3-1 in the rat model of LPS-induced lethality. We dosed the animals to give approximately the same peak concentration and area under

the curve for all three forms of EB3-1 between the period 2 and 4 hours after lethal LPS dosing. Thus, 4 groups of 10 rats received i.v. lethal doses of LPS at T=0. Then at T=2 hours all received EB3-1 forms as follows:

- 5 GROUP I - no therapy  
GROUP II - EB3-1 -- i.v. bolus 5 mg/kg at 1.0 ml/kg  
GROUP III - EB3-1 acid treated -- i.v.b.- 5 mg/kg  
GROUP IV - EB3-1 F(ab)'<sub>2</sub> - 7.5 mg/kg T=2 hours  
5.0 mg/kg T=3 hours  
10 2.5 mg/kg T=4 hours

This dosing regimen approximates the same area under the blood clearance curve as is obtained with unmodified EB3-1 at 5 mg/kg. Results, shown in Figure 7, suggest that the mechanism(s) of protection is not intricately involved with  
15 complement fixation. In addition, we have demonstrated ways of abrogating the potentially-toxic ability of this murine IgG3 to fix human complement and/or actuate cells by binding to F<sub>c</sub>-receptors.

20 Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for inhibiting intercellular adhesion mediated by ELAM-1 in a patient, the method comprising  
5 administering to the patient a therapeutically effective dose of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunoglobulin which recognizes a functional epitope on ELAM-1.
- 10 2. The method of claim 1, wherein the immunoglobulin is IgG<sub>3</sub>.
3. The method of claim 1 wherein the immunoglobulin is secreted by a cell line designated A.T.C.C. Accession No. HB  
15 10591.
4. The method of claim 1, wherein the intercellular adhesion is associated with an inflammatory response.
- 20 5. The method of claim 4, wherein the inflammatory response is septic shock.
6. The method of claim 4, wherein the inflammatory response is adult respiratory distress syndrome or wound  
25 associated sepsis.
7. The method of claim 1, wherein the intercellular adhesion is associated with metastasis.
- 30 8. The method of claim 1, wherein the pharmaceutical composition is administered intravenously.
9. The method of claim 8, wherein the therapeutically effective dose is between about 1 mg/kg body  
35 weight to about 20 mg/kg body weight.

10. The method of claim 8, wherein the therapeutically effective dose is between about 5 mg/kg body weight to about 15 mg/kg body weight.

5 11. A method of treating a disease response mediated by ELAM-1 in a patient the method comprising administering to the patient a therapeutically effective dose of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunoglobulin which recognizes a  
10 functional epitope on ELAM-1.

12. The method of claim 11, wherein the immunoglobulin is IgG<sub>3</sub>.

15 13. The method of claim 11, wherein the immunoglobulin is secreted by a cell line designated A.T.C.C. Accession No. HB 10591.

14. The method of claim 11, wherein the disease  
20 response is an inflammatory response.

15. The method of claim 11, wherein the disease response is septic shock.

25 16. The method of claim 11, wherein the disease response is adult respiratory distress syndrome or wound associated sepsis.

17. The method of claim 11, wherein the  
30 immunoglobulin is embedded in a liposome.

18. The method of claim 17, wherein the liposome encapsulates an anti-inflammatory chemotherapeutic agent.

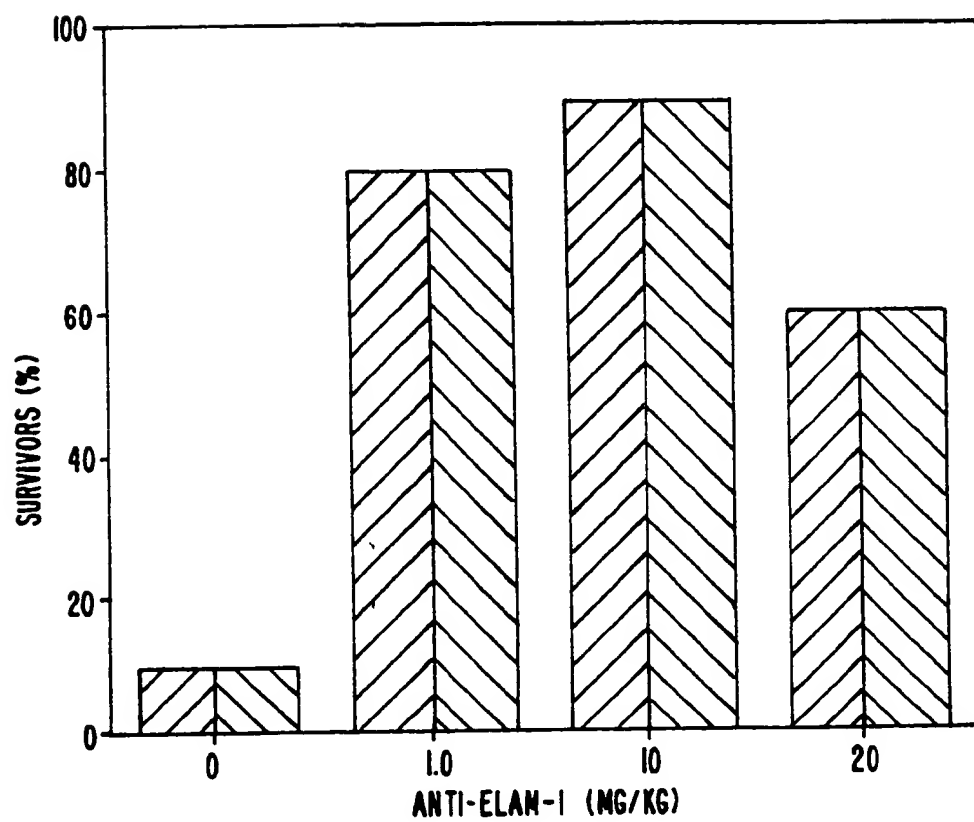
35 19. A pharmaceutical composition comprising an immunoglobulin capable of binding a functional epitope on ELAM-1 and inhibiting a disease response mediated by ELAM-1 in a patient.

20. The composition of claim 19, wherein the immunoglobulin is secreted by a cell line designated A.T.C.C. Accession No. HB 10591.

5           21. The composition of claim 19, wherein the disease response is septic shock.



1/5

 24 HOURS 7 DAYS**FIG. 1.**

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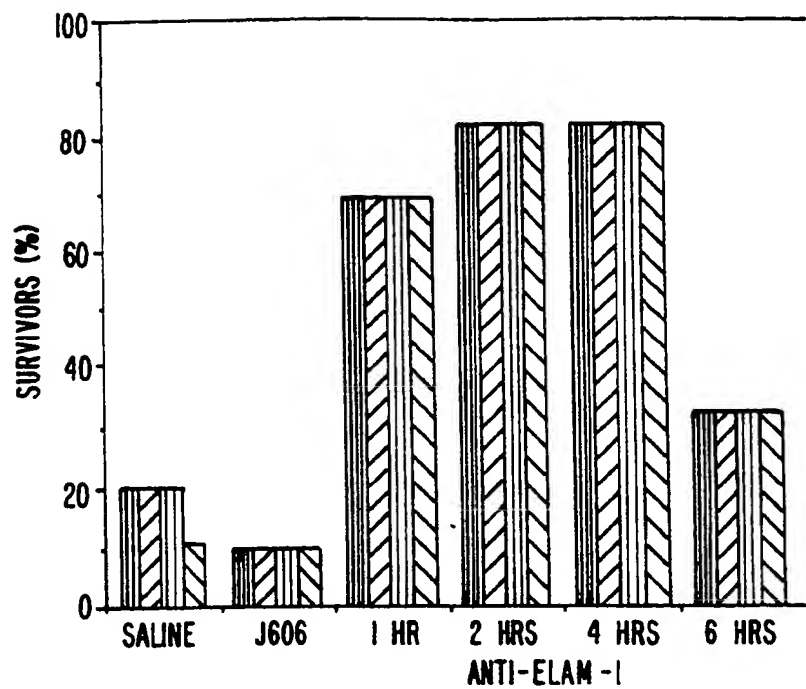


FIG. 2.

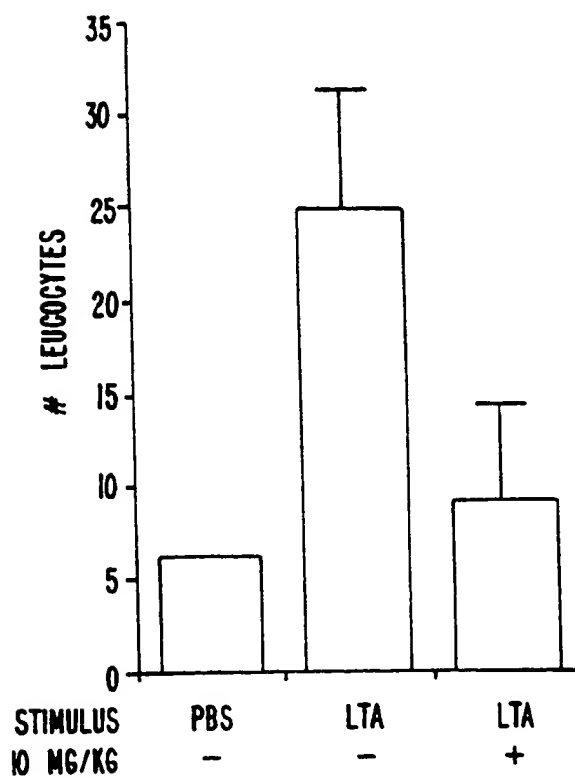


FIG. 3.

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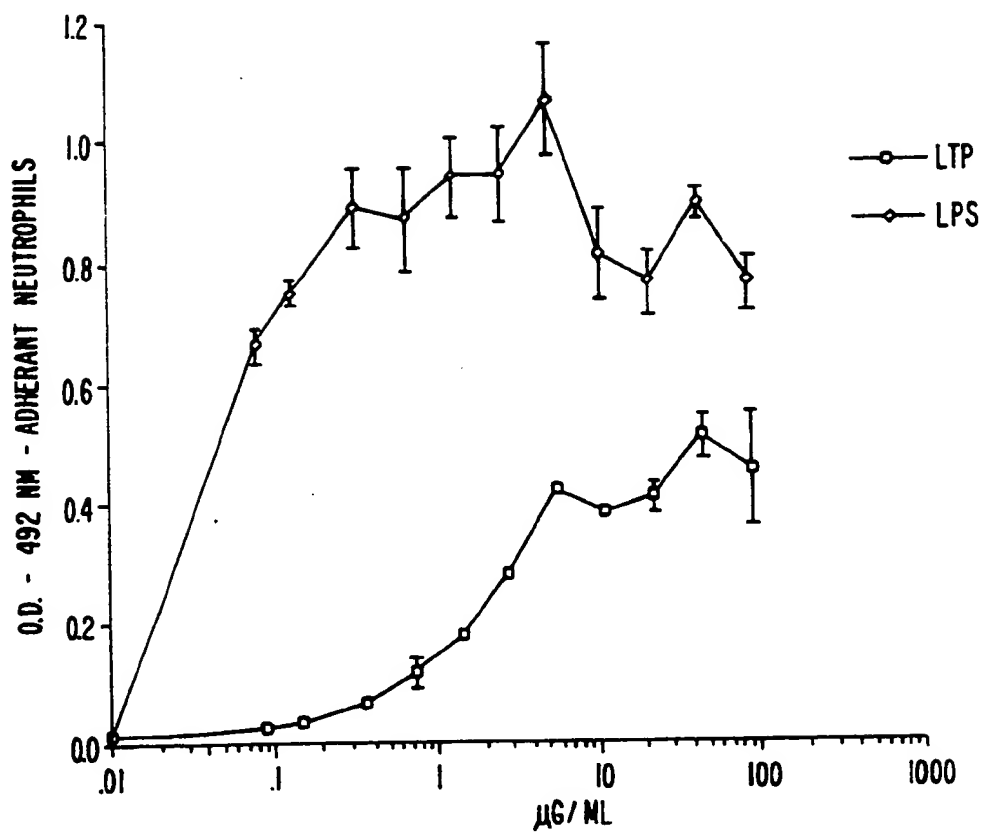


FIG. 4.

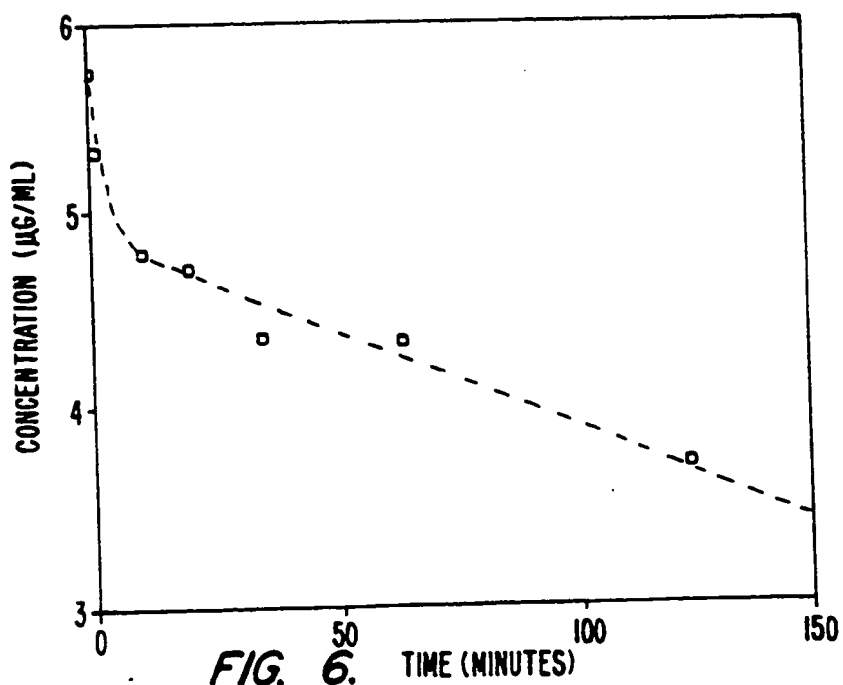


FIG. 6. TIME (MINUTES)

SUBSTITUTE SHEET

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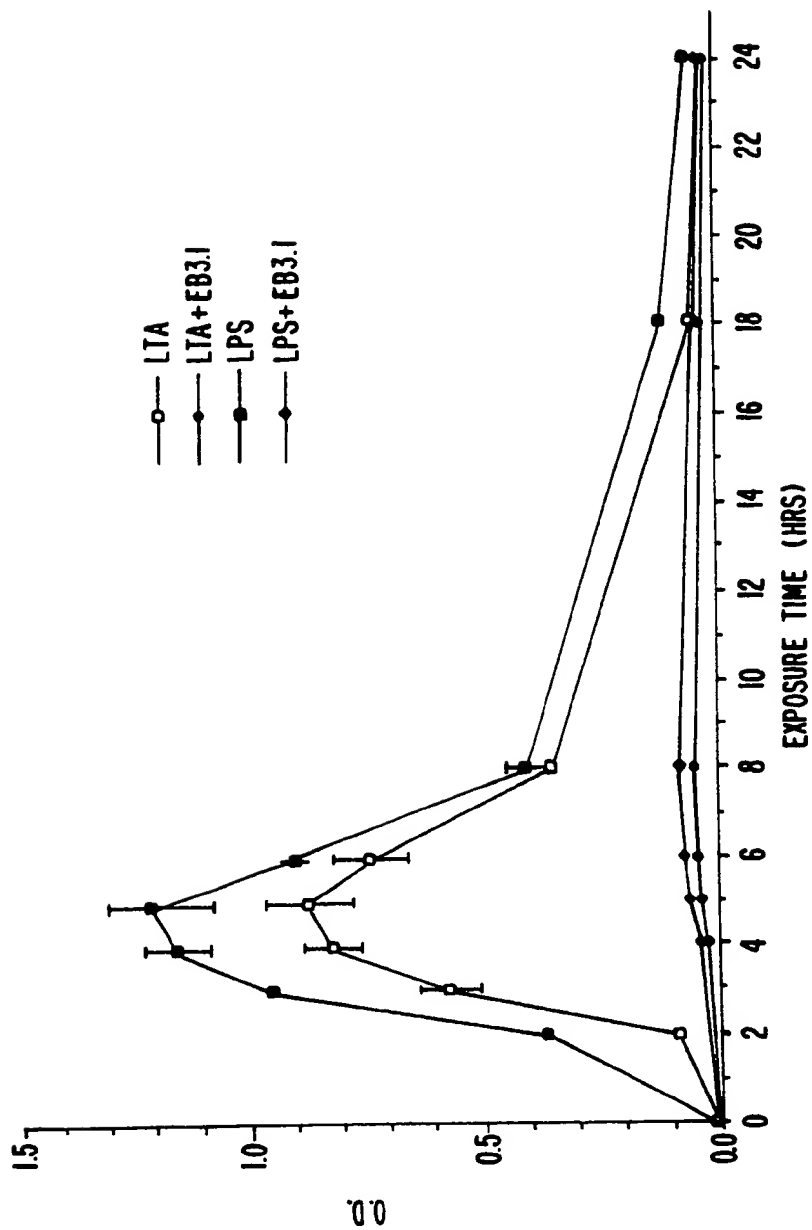
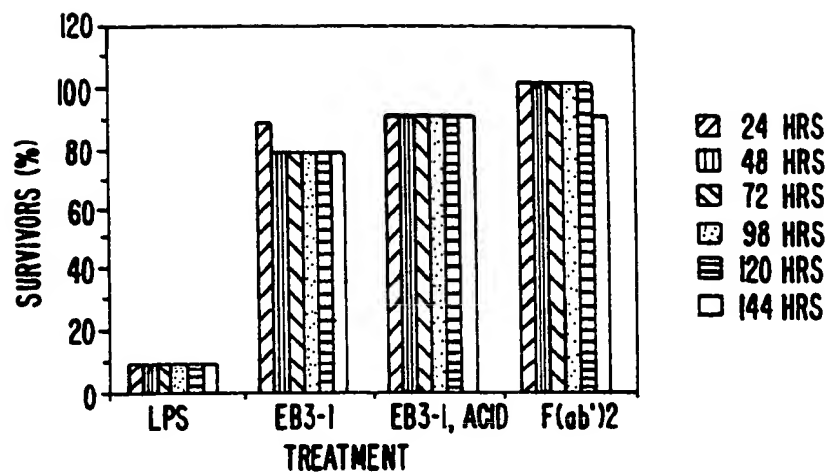


FIG. 5.

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*FIG. 7.*

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00577

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ( ) : A61K 37/22, 39/00; C07K 17/00 US CL : 424/85.8, 450; 435/70.21, 172.2, 240.27; 530/387		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
	424/85.8, 450; 435/70.21, 172.2, 240.27; 530/387	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
APS, BIOSIS, CAS, MEDLINE, WPI search terms: ELAM 1, selectin, inflamm?, antibod?, wayner, phillips		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>1</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
y	WO, A, 90/05539 (Bevilacqua et al.) 31 May 1990, see entire document, particularly pages 11, 17 and 22-26.	1-21
y	Science, Volume 243, issued 03 March 1989 M.P. Bevilacqua et al., "Endothelial Leukocyte Adhesion Molecule 1: An Inducible Receptor for Neutrophils Related to Complement Regulatory Proteins and Lectins", pages 1160-1165, see entire document.	1-21
y	Science, Volume 250, issued 23 November 1990, M.L. Phillips et al., "ELAM-1 Mediates Cell Adhesion by Recognition of a Carbohydrate Ligand, Sialyl-LeX", pages 1130-1135, see entire document.	1-21
y,p	Proc. Natl. Acad. Sci. (U.S.A.), Volume 88, issued 01 July 1991, M.J. Polley et al., "CD62 and Endothelial Cell-Leukocyte Adhesion Molecule 1 (ELAM-1) Recognize the Same Carbohydrate Ligand, Sialyl-Lewis X", pages 6224-6228, see entire document particularly Figure 5.	1-21
<p>* Special categories of cited documents:<sup>15</sup></p> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>2</sup>
21 APRIL 1992		30 APR 1992
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>20</sup>
ISA/US		Phillip Gambel

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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
y	Immunological Reviews, Volume 14, issued April 1990, T.M. Carlos et al., "Membrane Proteins Involved in Phagocyte Adherence to Endothelium", pages 5-28, see pages 10-13 and 19-20.	1-21
<b>V. <input type="checkbox"/> OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup></b> This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons: 1. <input type="checkbox"/> Claim numbers __, because they relate to subject matter (1) not required to be searched by this Authority, namely:  2. <input type="checkbox"/> Claim numbers __, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:  3. <input type="checkbox"/> Claim numbers __, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).		
<b>VI. <input type="checkbox"/> OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup></b> This International Searching Authority found multiple inventions in this international application as follows:  1. <input type="checkbox"/> As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. 2. <input type="checkbox"/> As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:  3. <input type="checkbox"/> No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:  4. <input type="checkbox"/> As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee. Remark on protest <input type="checkbox"/> The additional search fees were accompanied by applicant's protest. <input type="checkbox"/> No protest accompanied the payment of additional search fees.		

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